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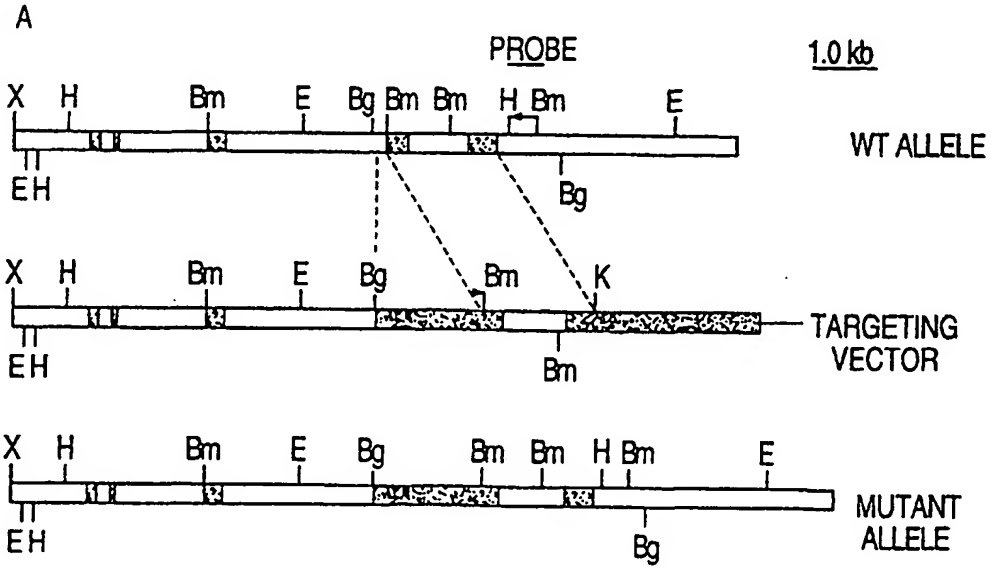

(57) Abstract

In vivo assays for inhibitors of human allergic responses employ humanized transgenic mice in which at least one human gene encoding a chain of the FcεRI receptor, replaces expression of its murine homologue. These assays are particularly useful for detecting inhibitors of IgE-mediated allergic responses.

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IN VIVO ASSAYS FOR INHIBITORS OF IgE MEDIATED ALLERGIC RESPONSES

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BACKGROUND OF THE INVENTION

A mouse line is established in which murine genes encoding structural proteins that are essential for an allergic response are replaced with their human counterparts, to provide an *in vivo* biological assay for inhibitors of an IgE-mediated human allergic response.

A Mammalian Allergic Response

A mammalian allergic response is a complex phenomenon resulting from the action of many genes encoding a plurality of structural and functional proteins. Two classical models of allergic reactions are cutaneous and systemic anaphylaxis (Klein, 1982).

Although the full mysteries of an allergic response still remain to be revealed, much information is now available about certain pathways and structural components leading to the manifestations of a suspected allergic response. It is of major importance for developing treatments for allergies, to understand the allergic response so that it can be controlled, for example, by inhibitors.

Many lines of evidence have led to the concept that the interaction between IgE and mast cells and basophils is the primary effector pathway in allergic responses. Mast cell activation occurs by cross-linking cell surface bound IgE with multivalent allergens, resulting in the release of mediators, such as histamine, which directly produce allergic symptoms (Galli, 1993; Ishizaka, 1989; Serafin and Austen, 1987). Mouse mast cells express at least three types of IgE receptor: the tetrameric high affinity IgE receptor (FcϵRI) which binds monomeric IgE (Kinet, 1992a; Metzger, 1992), and the two low affinity IgG receptors (FcγRII and III), which bind both IgG- and

IgE-immune complexes (Takizawa *et al.*, 1992). Two other IgE-binding cell surface structures, which are part of the lectin-like receptor family, have been identified: FcεRII/CD23 (Conrad, 1990) and Mac-2 (CPB35, εBP) (Cherayil *et al.*,1989; Frigeri and Liu, 1992; Truong *et al.*, 1993). Both are found on a large variety of hematopoietic cells. In addition, FcεRI is not only expressed on mast cells and basophils, but also on Langerhans cells (Wang *et al.*, 1992; Bieber *et al.*, 1992) and monocytes(Maurer *et al.*, 1993). Thus, there are many hematopoietic cells capable of binding IgE and a variety of IgE-binding structures. The relative role of these IgE receptors in IgE-mediated allergic reactions is currently unknown.

In addition to mediating degranulation, FcεRI initiates the release of cytokines, such as interleukin-3, a mast cell growth factor (Burd *et al.*, 1989; Plaut *et al.*, 1989; Wodnar-Filipowicz *et al.*, 1989; Galli,1993). FcεRI expression occurs early in ontogeny, at stages where mast cells are not yet granulated (Thompson *et al.*, 1990). In these precursor cells,FcεRI cannot mediate degranulation, but it may participate in cytokine secretion and play an as yet undefined role in mast cell development.

The IgE High Affinity Receptor

Mast cells and basophils, which are activated by immunoglobulin E (IgE) and allergen, play a prominent role in anaphylaxis. However, these cells express at least three types of IgE receptor,including the high affinity IgE receptor (FcεRI). One of the proteins believed to be involved in an allergic response is the FcεRI receptor. The relative contribution of this receptor, and possibly other receptors such as CD23/FcεRII and Mac-2, to the genesis of in vivo anaphylaxis is still unclear.

A long unanswered question is whether FcεRI or other IgE-binding structures such as FcγRII, FcγRIII, CD23/FcεRII or Mac-2 are causally involved in

IgE-dependent anaphylactic responses. In addition, other unknown mechanisms could also play a critical role.

Because IgE appears to confer protection against some parasites (Hogan *et al*, 1991, Rihet *et al.*, 1991, Dunn *et al.*, 1992), the FcεRI receptor may be involved in this protection.

Assay for Inhibitors of an Allergic Response

The search for inhibitors of an allergic response is hampered by lack of availability of accurate, inexpensive and rapid assays. Inhibitors of allergic responses may be detected by *in vitro* assays, or *in vivo* assays.

***In Vivo* versus *In Vitro* Assays**

In vivo assays are needed to follow up *in vitro* screening for inhibitors. This is true because the whole animal systematic interactions are not completely predictable from *in vitro* studies, which cannot totally model complex immune responses. Accordingly, it is dangerous and, in fact, prohibited to proceed directly from *in vitro* assays to clinical trials.

Candidate inhibitors are tested in whole animals, for example, to determine if they block anaphylaxis. However, inhibition of an animal system may not be predictive of effects in humans. Also, these models do not provide information on mechanisms of inhibition to allow zoning in on the design of inhibitors of specific targets.

Transgenic Mice as *In Vivo* Assays

To move up the scale of complexity from *in vitro* to *in vivo* assays, small mammals which are models of the human allergic response system are preferable. To provide a useful model, mice ideally produce a "human-like response." This would be facilitated if human transgenes were present.

The first transgenic mouse was patented in 1987 (the "Onco Mouse™"). A few other types of transgenic mice have been produced (U.S. Pat. No. 5,175,383 and 5,175,385), but are not suitable for studies of the allergic response. Limitations of transgenic mice include random and unpredictable incorporation of transgenes into a lost genome. Location of incorporation and number of copies have generally not been controlled. Transgenic mice that have been "humanized" that is, had sections of their genome **replaced** by a human homologue, are not available.

SUMMARY OF THE INVENTION

Pursuant to the present invention, an *in vivo* assay for inhibitors of IgE-mediated allergic response makes use of transgenic mice in which a human gene encoding at least one chain of the FcεRI replaces its murine homologue. These are "humanized" mice, not merely mice into which a human transgene is introduced. To develop such a transgenic mouse strain, a "knockout" mouse is produced in which at least one gene encoding an FcεRI chain is rendered non-functional. To obtain the "humanized" transgenic mice of the present invention, a human gene that is substantially homologous, replaces the murine gene in the FcεRI-deficient mice. Preferably, the human gene is incorporated into the genome in the same position as was its murine counterpart.

FcεRI-deficient mice provide useful *in vivo* models with which to address questions regarding the physiological role of FcεRI so that effective inhibitors of deleterious allergic responses may be developed. The plethora of IgE-binding proteins present *in vivo*, along with coexpression of such proteins on many immune cells, previously has made it essentially impossible to dissect out individual roles for each protein involved in IgE-mediated immune responses. Using FcεRI deficient mice according to the present invention provides an opportunity to characterize the FcεRI -/- phenotype, as well as to address questions about the role of FcεRI in

initiating anaphylactic responses and in mast cell ontogeny. Preferred mammals in this context are mice. Such detailed knowledge facilitates choice and design of candidate inhibitors of the allergic response.

Transgenic mice are used to reconstitute a receptor that is a model for the human receptor. Such mice are prepared by incorporating the human gene encoding the α chain of the IgE receptor into the strain of mice homozygous for a disrupted α chain gene.

According to the present invention, transgenic mice that express only the human and not the murine IgE receptor are produced by incorporating into mice with a disrupted FcεRI chain gene, the homologous gene encoding the human chain. Incorporation may be effected by breeding the FcεRI-deficient mice to mice having the human transgene. Crossing-over and recombination (phenomena which occur predictably during meiosis) yields transgenic mice which have the human gene substituted for the murine gene in the mouse genome. Preferably,the murine promoters are regulating expression of the human counterpart. Other suitable promoters include CMV and HTLV1.

In an illustrative embodiment, a strain of mice is established in which the gene encoding the α chain of the high affinity IgE receptor is disrupted by homologous recombination. The result of disruption of the α chain gene in this manner is to abolish the surface expression of the IgE receptor on mast cells. Surprisingly, however, mast cell development proceeds in a normal pattern, despite disruption of one of its cell surface proteins.

To produce a strain of mice deficient for FcεRI expression, a targeting plasmid is designed and constructed to disrupt the fourth exon of the FcεRI gene. This region is preferred for disruption because it is one of the two exons encoding the two Ig domains. For practice of the present invention, the third exon of the FcεRIα gene is also suitable for disruption by homologous

recombination. The gene encoding the β chain also is suitable for disruption. Preferably, both the genes encoding the alpha and the β chain are disrupted.

The targeted plasmid is incorporated into mice via mouse embryonic stem cells by electroporation or calcium phosphate precipitation.

Mice homozygous for the disrupted alpha gene do not have any overt phenotype. These mice are resistant to both cutaneous and systemic anaphylaxis, demonstrating that the high affinity IgE receptor is necessary for *in vivo* IgE dependant anaphylaxis. Therefore, interfering with the structure, and thereby the function of the receptor provides an effective means of treating an allergy regardless of the allergen specificity. This generalized treatment is possible because all allergic responses use the IgE receptor as a conduit.

These transgenic mice are useful as *in vivo* assays of inhibitors of human allergic reactions, because they express the high affinity IgE receptor only if the human chains of the receptor are produced. Otherwise, because there is no receptor, there is no response.

According to the present invention, therefore, an *in vivo* assay for an allergic response includes the following steps:

(i) providing a transgenic mouse that expresses at least one human gene encoding a chain of the FcεRI receptor substituted for the homologous mouse gene (a "humanized" transgenic mouse) and

(ii) subjecting said transgenic mouse to conditions conducive to an allergic reaction and then

(iii) detecting whether said allergic reaction occurred.

The presence of an allergic response in the mice is detectable, for example, by inducing passive cutaneous anaphylaxis or systemic anaphylaxis.

In an assay of the present invention, the substituted

human gene encodes the alpha chain of the FcεRI receptor or the beta chain. Two genes, one encoding the alpha and one the beta chain, also are suitable.

Pursuant to another aspect of the present invention, an *in vivo* method to screen for an agent that inhibits an allergic response in a human includes the following steps:

(a) exposing a humanized transgenic mouse to said agent, wherein said transgenic mouse expresses at least one human gene encoding for a chain of the FcεRI,

(b) subjecting said humanized transgenic mouse to conditions conducive to an allergic reaction and then

(c) determining whether an allergic reaction occurs, whereby any inhibition of said allergic reaction by said agent is gauged.

Pursuant to yet another aspect of the present invention, a method of affecting an allergic response entails treating a patient with an amount of an inhibitor which is determined, via an assay of the present invention, to be sufficient to inhibit said response.

In contrast to the present invention, a transgenic mouse with a human gene superimposed on a complete murine system is not preferred because (1) unexpected interactions are possible when both murine and human genes are encoding a similar chain; (2) competition between the human and mouse α chains for β and γ chains is likely to decrease receptor expression; this could produce false negative results in an inhibitor assay; (3) human binding of the high affinity receptor in the presence of the mouse high affinity receptor is not known.

Candidate substances that may be capable of inhibiting an allergic response are selected for screening. Likely candidates include peptides and small, charged molecules that are not capable of entering a cell. These candidate substances are then screened,

generally by *in vitro* assays, to select candidates for *in vivo* testing. The *in vivo* inhibitor assays of the present invention are used after a random *in vitro* screen has identified potential candidate inhibitors.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. A restriction map of a targeting vector construct and of a wild-type and mutated allele is shown. Exons are indicated by black boxes; *neo* and thymidine kinase cassettes are represented by hatched boxes. Bg, Bm, E, H, K and X represent restriction sites for BgIII, BamHI, EcoRI, HindIII Kpnl and Xhol respectively. PCR primers are indicated by arrowheads, and a probe used to detect homologous recombination events is shown.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

It has been discovered that an intact FcεRI is necessary for an IgE-mediated response, and that an inhibitor of any component of FcεRI will prevent an allergic response. According to the present invention, therefore, *in vivo* assays for inhibitors of human allergic responses employ humanized transgenic mice in which at least one human gene encoding a chain of the OFcεRI receptor replaces its murine homologue. Such assays are particularly useful for detecting inhibitors of IgE-mediated allergic responses, *i.e.*, inhibitors of IgE binding to the receptor.

FcεRI is a tetrameric complex of one α, one ß, and two disulfide-linked γ chains (Kinet, 1992a; Metzger, 1992). Because only the fully assembled complex is expressed on the cell surface (Blank *et al.*, 1989), targeting one subunit is a strategy to prevent FcεRI expression. Because γ subunits are part of other Fc receptors and of the T cell antigen receptor (Ravetch and Kinet, 1991), and play a critical role in signal transduction (Kinet, 1992b; Weiss, 1993), targeting γ affects the expression and function of receptors other than FcεRI. For this reason, and because the receptor binding site is contained entirely in the extracellular portion of α (Hakimi *et al.*, 1990; Blank *et al.*, 1989;

Robertson,1993), the α subunit gene is preferably disrupted and replaced with the human homologue. A cDNA sequence encoding the α subunit of the human mast call IgE surface receptor, and its use in recombinant genetic production of α subunits, are disclosed in U.S. Pat. No. 7,127,214.

The ß chain is also a suitable target for disruption and replacement by the human ß chain encoding gene (Kuster *et al.*, 1992). In a preferred embodiment, both the α and ß chain genes are disrupted and replaced with active human counterparts. "Active" means the genes are capable of expression under suitable regulatory control.

Using methods of the present invention, mice homozygous for a disrupted α chain gene have been found not to express cell surface FcεRI. Thus, investigations using these mice demonstrated that FcεRI does not play a major role in mast cell development. Yet the presence of intact FcεRI is necessary to induce local or systemic IgE-mediated anaphylaxis. Therefore, inhibitors of anaphylaxis can be detected by disruptive effects on FcεRI.

FcεRI-deficient mice are completely protected from IgE-mediated anaphylaxis. This is true despite the fact that these mice express higher levels of FcγRII/III and that experimental conditions were used where one might expect the formation of IgE-immune complexes which could activate low affinity IgE-binding proteins. Therefore, FcεRI is necessary for the induction of IgE-mediated anaphylaxis, with a minor role at most for the other lower affinity IgE-binding proteins. The role of IgG immune complexes also seems to be minimal, because systemic anaphylactic reactions could not be induced in any mice, either FcεRI +/+ or -/-, even after intravenous injection of 0.4 mg of IgG. Other IgE-binding proteins may play a role if alternative methods, such as active immunization (Martin *et al.*, 1993), are used to induce antigen sensitivity. FcεRI clearly is necessary, however, for initiating the cascade of events leading to

the various symptoms of IgE-mediated anaphylaxis. Accordingly, targeting FcεRI is an effective way to prevent or treat allergic reactions.

Although the evidence for a protective role of IgE in parasitic infestations is mixed, IgE seems to be protective against chronic infestations of *Schistosoma* in humans (Hagan *et al.*, 1991; Rihet *et al.*, 1991; Dunne *et al.*, 1992), implicating FcεRI in the mechanisms of protection against parasites. The FcεRI-deficient mice are useful to determine whether FcεRI is necessary for protection against parasites. If it is, compositions enhancing this protection may be developed by use of the humanized transgenic mice of the present invention.

Generation of FcεRI (-/-) Mice

The term "FcεRI (-/-)" is used in this description to denote herein a genotype responsible for a phenotype wherein the IgE high affinity receptor is not expressed. This includes genotypes FcεRIα (-/-) which may be specified to indicate that a gene encoding the α chain is non-functional (not expressed as an α chain).

Although methods to produce "knockout" mice are reported, many problems and uncertainties face the approach (Travis, 1992).

Several uncertainties are associated *a priori* with production of an FcεRI-deficient mouse line within the present invention. For example, an important consideration in attempting genome alteration by gene targeting is whether a given targeting plasmid will actually give rise to the desired phenotype. Plasmids used for gene targeting are often designed to inactivate a specific gene by the introduction of a "null mutation," that is, a mutation which completely destroys the ability of a gene to produce a functional protein. Many of these targeting plasmids are designed to introduce null mutations by inserting exogenous sequences into the center of a gene, thus interrupting the normal protein coding sequence. This strategy has failed in a number of cases, however, because of the ability of the cellular

transcriptional machinery to remove the RNA encoded by the exogenous sequences during splicing.

To avoid this situation, a targeting plasmid is preferably designed that replaces coding sequences necessary for the function of the encoded protein with exogenous sequences. One obstacle that is encountered and must be overcome in designing a suitable targeting plasmid pertains to the small size of the genes encoding FcεRI subunits, notably the α chain gene, which limits the number of endogenous restriction sites that are suitable for use in constructing a targeting plasmid that would produce the expected null phenotype. A way to circumvent this problem is to use PCR technology to generate the smaller of the two arms of homologous DNA that direct the targeting event. This approach provides maximum flexibility in choosing the length and location of the homologous DNA and the position in which the gene is disrupted. The length of the smaller arm is determined by balancing the need for a reasonable targeting frequency, which increases with the length of homology, with the need to produce the arm by PCR, for which efficiency increases as the length of the reaction product decreases. To further increase chances of finding targeting events, isogenic DNA is used to construct this arm. This increases the probability that there are no differences between the target locus and the targeting construct.

In designing a targeting plasmid, it also is important to determine how targeting events will be distinguished from instances in which the targeting plasmid integrates randomly into the genome. To maximize chances of successfully screening for targeting events by PCR, a targeting plasmid is constructed by modifying a known plasmid (see next section) by the addition of a linker sequence. This sequence is preferably one that is believed to ensure a good primer binding site for PCR across the DNA corresponding to the short arm of the targeting plasmid in the targeted cell lines. In

choosing the regions of homology included in the targeting plasmid, attention is also paid to the need to isolate genomic probes for verification of targeting events, for example, by Southern analysis.

Another uncertainty that is taken into consideration in carrying out the targeting experiments is the relative targeting frequency of individual targeting experiments. To monitor this parameter, a control electroporation is carried out in parallel with each experimental electroporation, where electroporation is used to incorporate the targeting plasmid into a host cell. For these control electroporations, a construct designed to target a marker such as the hprt locus is suitable. This permits determination of the targeting frequency for a well characterized locus before screening for targeting events at the experimental locus. Thus, it is possible to anticipate whether a targeting event for the FcεRI locus is likely to be identified in a given experiment.

The choice of a targeting cell line also is important in the success of the targeting experiments. For example, three reasons motivated the choice of the E14TG2A cell line in the illustrative examples below. First, the *hprt* gene in this cell line is inactive, making it possible to carry out the control electroporations described above. Second, because E14TG2A is derived from the 129/Ola strain, rather than the 129/Sv strain from which most mouse ES cell lines are derived, it carries two recessive mutations, pinkeyed and c^{ch}. The presence of these genes which produce readily distinguishable phenotypes, make it possible to obtain light coated mice in the first generation, enabling identification of potential recombinants for many of the assays critical for characterizing this animal to be carried out readily (e.g. to determine whether the murine α chain is disrupted). Finally, the absence of the inactivated *hprt* gene in this cell line allows hprt to be used as a selectable maker to further modify these targeted cells. This is very important because this

targeting is the first of a series of steps necessary for generation of a mouse humanized in terms of its IgE response.

A final factor that plays a role in the production of the targeted mice is the breeding scheme used to obtain mice homozygous for the targeted locus. To minimize the breeding time required to obtain these homozygous animals, chimeric males that transmit the ES cell genome to large percentages of their offspring in their first litters were bred to their own daughters (e.g. in the illustrative examples herein, mice were selected that transmitted the ES cell genome to at least 75% of their offspring).

The FcεRIα subunit is a type I transmembrane molecule with an extracellular segment containing two immunoglobulin (Ig) related domains, the second of which is necessary for IgE binding (Robertson, 1993). The FcεRIα gene comprises five exons, with the third and fourth exons encoding the two Ig domains (Tepler *et al.* 1989; Ye al., 1992). A targeting plasmid is preferably designed to disrupt the fourth exon by insertion of a neomycin gene (an illustrative construction is shown in FIG. 1). In the example shown, after electroporation with this DNA, D3 and E14TG2 embryonic stem cells (ES) were selected in the presence of G418 and gancyclovir to enrich for those that had integrated the vector by homologous recombination. Of 600 colonies isolated and expanded, at least nine were scored positive for targeted integration of the vector by polymerase chain reaction (PCR) and by Southern Blot analysis, demonstrating integration via homologous recombination at the FcεRIα locus. Hybridization of the same blots with a *neo* probe showed a single copy of the *neo* gene. ES cells from three of these lines were injected into C57BL/6 blastocysts. The chimeras generated from two of these cell lines transmitted the mutant gene to their offspring. This was demonstrated by Southern Blot analysis of mouse tail DNA which showed hybridization

with a 6.1 kb wild type allele fragment and/or a 7.7 kb fragment for the mutated (alpha chain gene deficient) allele. No overt phenotype was observed in animals that were either heterozygous or homozygous for the mutant gene. Heterozygous and homozygous animals were present in litters at the expected ratios.

Construction of the Targeted Plasmid

The targeting vector is constructed by cloning two fragments of the mouse FcεRIα into a vector such as pJNS2. The pJNS2 vector is derived by inserting a 50 bp polylinker between the XbaI and BamHI sites of pNT2 (Tybulewicz *et al.* 1991). A genomic clone is isolated by screening, for example, a 129/Ola genomic lambda phage library with mFcεRIα cDNA (Ra *et al.*, 1989). A 1.6 kb BglII-KpnI fragment encompassing exons IV and V is generated by PCR of the genomic clone with primers C A G G T A C C C A G T G T T T A T T G A G and GAAGATCTGGATCCTTTGACATCAGATGCC, then inserted between the BamHI and KpnI sites of pJNS2 vector, 3' to the *neo* gene and 5' to the TK gene. A second fragment, a 5.5kb XhoI-BglII fragment including exons I to III is available from a Balb/c library genomic clone (Ye *et al.*, 1992), subcloned in pSL301 (In Vitrogen), then excised by digestion with XhoI and SalI and inserted into the XhoI site 5' of the *neo* gene of pJNS2. Thus, the final targeting construct is identical to the corresponding region in the mouse genome, except that a 0.3 kb BglII-BamHI fragment that encompasses the 3' end of intron III and the 5' end of exon IV has been replaced by the positively selectable *neo* gene in pJNS2.

ES Cell Culture, Electroporation and Selection of ES Cells

There are many ways known to those of skill in the art to transfer genetic material into a host. One way is to electroporate mouse embryonic stem cells. This method is described in the following section.

In an illustrative embodiment, the ES cell lines D3 (Doetschman *et al.*, 1987) and E14TG2a (Hooper *et al.*,

1987) are cultured as previously described (Koller and Smithies, 1989). Electroporation is carried out in culture medium containing 3nM of targeting plasmid (linearized with NotI), using conditions described previously, except that a 1-sec discharge from a 150-250 mF capacitor charged to 250-400 V is used. Transfected colonies are selected by growth in G418 and enriched for targeted colonies by the addition of gancyclovir (a gift from Syntex, Palo Alto, CA) to the growth medium. Surviving colonies are expanded to a level sufficient for PCR and Southern blot analysis.

The targeting vector is constructed by cloning two fragments of the FcεRIα gene into the vector pJNS2. The pJNS2 vector is derived by inserting a 50 bp polylinker between the Xba I and Bam HI sites of pNT2 (Tybulewicz *et al.*, 1991). A genomic clone including the FcεRIα gene is isolated by screening a 129/Ola genomic lambda library with the mFcεRIα cDNA. A 1.6 kb Bgl II-Kpn I fragment encompassing exons IV and V is generated by PCR of the genomic clone with primers CAGGTACCCAGTGTTTATTGAG and GAAGATCTGGATCCTTTGACATCAGATGCC and then inserted between the Bam HI and Kpn I sites of the pJNS2 vector, 3' to the *neo* gene and 5' to the tk gene. A second fragment, a 5.5 kb Xho I-Bgl II fragment includes exons 1-3 and is available from a BALB/c library genomic clone, subcloned in pSL301 (In Vitrogen) and then excised by digestion with Xho I and Sal I and inserted into the Xho I site 5' of the *neo* gene of pJNS2. The final targeting construct is identical to the corresponding region in the mouse genome, except that a 300 bp Bgl II-Bam HI fragment that encompasses the 3' end of intron 3 and the 5' end of exon 4 is replaced by the positively selectable *neo* gene in pJNS2.

The ES cell lines, E14TG2a (Hooper *et al.*, 1987) and D3 (Doetschman *et al.*, 1987), are cultured on irradiated primary embryonic fibroblast-feeder layers essentially as described by Koller and Smithies (1989). Neomycin resistent embryonic fibroblasts are prepared from embryos

from $\beta 2(-/-)$ females that had mated 14 to 17 days earlier. These mice contain a copy of a neomycin gene within the $\beta 2$-microglobulin locus and thus are capable of growth in media containing G418. Electroporation conditions are similar to those that have been described previously. ES cells are trypsinized, resuspended in culture media at a concentration of 4×10^7/ml and electroporated in the presence of the targeting DNA at a concentration of 12 nM DNA in a first experiment and 5 nm in a second. A voltage of 300 V and capacitance of 150-250 mF are optimal for an electroporation cell of 5 mm length and 100 mm^2 cross section. 5×10^6 electroporated cells are plated onto irradiated fibroblasts in 100 mm dishes in the presence of Dulbecco's modified Eagle's media (DMEM) supplemented with 15% fetal bovine serum (FBS) and 0.1 mM 2-mercaptoethanol. The media is replaced 24 hr after electroporation with media containing 200 μg/ml G418 and 1 μM ganciclovir

In an example, three independent targeting experiments were carried out. These experiments were called EP65, EP66 and EP67. In each, $2x\ 10^7$ E14TG2a cells were electroporated in the presence of the FcϵRIα targeting plasmid, which was linearized by restriction with Not I. Cells were cultured in G418 and ganciclovir. All experiments were identical, except that in EP67 $2x10^7$ D3 cells were used. ES cell lines 66-154, 66-161, 66-168, 66-196, 67-327, 67-490, 67-501, 67-502, 67-602 were identified as carrying a targeted FcϵRIα locus.

Analysis of G418 and Gancyclovir Resistant ES Cell Colonies

ES cell colonies visible 10-14 days after electroporation are picked with drawn out capillary pipettes into 24 well plates that are seeded with irradiated feeders. After sufficient growth (usually two to three days) wells are trypsinized and cells moved to 60mm wells seeded with feeder cells. When plates become confluent, cells are again trypsinized, and one half of the cells are frozen using conditions which have

been found to be optimal for recovery of embryonic stem cells. DNA is prepared from the remaining cells and used to screen for targeting events by the polymerase chain reaction (PCR) or by Southern blotting.

DNA is prepared following a previously described method (Miller *et al.*, 1988). Essentially, cells are resuspended in 300μl buffer containing 0.05M Tris(pH8), 0.1M NaCl, 0.1M EDTA, 1 % SDS and 150μg of Protinase K. After incubation for 24 hr at 55 degrees, 180 μl of a saturated NaCl solution is added. Preparations are centrifuged and supernates removed. DNA is then precipitated by the addition of two volumes of ethanol. DNA is resuspended in Tris EDTA buffer (10mm Tris pH 7.5, 1mm EDTA) at approximately 1μg/μl concentration.

For PCR screening, primers are chosen which hybridize to the pJNS2 polylinker (bases 2-35) and to a region 150 bp 3' from exon 5. (GCTTCTAGCAACAGAAGGCAGATTAC). The length of the expected product is 1.75 kb for cases of homologous recombination. There is no amplification in cases of random integration.

Southern Blot and PCR Analysis

Methods suitable to confirm disruption of a gene encoding α chain of the FCεRI receptor are employed. These methods include Southern analysis and PCR. For Southern analysis in an illustrative embodiment, DNA is digested with restriction enzymes as directed by the manufacturers, and fragments are separated on 0.7% agarose gels. DNA is transferred to nylon membranes and probed with a ^{32}P labelled 350 bp Hind III-Bam HI fragment that is located 150 bp downstream of exon 5. The hybridization pattern obtained with the restriction enzyme Eco RI is expected to be altered by integration of the targeting plasmid into the endogenous FcεRIα locus by homologous recombination. For genomic DNA from targeted as well as non-targeted cell lines, it is expected that the probe hybridizes to an EcoRI fragment of approximately 6.1kb derived from the endogenous locus. However, for targeted lines, the intensity of the band

representing this fragment is expected to be reduced by 50%, and an additional band, generated by homologous recombination of the targeting plasmid with the FcεRIα locus, is expected to be present. The replacement of the 300 bp Bgl II-BamHI fragment of the FcεRI α gene with the 1849 bp neomycin insert in the alpha locus results in an EcoRI fragment that is approximately 1.5kb longer than the corresponding fragment in the native locus.

In an example, rehybridizing the filters used for detection of targeting events with a probe for the neomycin gene, it was demonstrated that additional copies of the neomycin gene had not integrated into the genome of targeted lines outside of the targeted locus. Targeted lines 66-154, 66-161, 66-168, and 66-196 were identified from experiment EP66, while lines 67-327, 67-490, 67-501, 67-502, and 67-602 were identified from experiment EP67.

Embryo Manipulation and Blastocyst Injection

After the confirmation that homologous recombination has occurred to render the target gene of the host cell non-functional, the transformed cell is used to generate a new mouse strain. This may be accomplished by injecting a mouse blastocyst with the cell. In an illustrative embodiment, the ES cell lines carrying the inactivated alpha gene are expected to allow the introduction of this mutation into the mouse germ line. Towards this end the following procedures are carried out. Mice may be purchased from Jackson Laboratories (Bar Harbor, ME). Blastocysts are obtained from 3 to 4 week old superovulated females, in this embodiment, they are from C57BL/6 mice. Uteri are flushed with M2 media 3.5 days after ovulation. Blastocysts are collected, washed several times in fresh M2 media, and placed in a 100 ml droplet of M2 under paraffin oil. ES cells identified as having the inactivated alpha locus are trypsinized, washed once with fresh DMEM media and diluted to approximately 2 x 10^6 cells/ml. 5 ml of cells are added to the droplet containing the blastocysts. 10

to 15 ES cells are injected into the blastocoel of each blastocyst using standard manipulation techniques.

Following injection, 6 to 9 blastocysts are returned to each uterine horn of pseudopregnant females mated 2.5 days previously with vasectomized males. Development of the blastocysts continued *in vivo* and pups are born about 16-18 days later.

In an example, C57BL/6 x DBA F_1 mice proved to be excellent foster mothers, because they yielded a pregnancy rate close to 100% and were able to raise small litters

The contribution of the ES cells to the offspring is judged visually by examination of the coat color of the pups if coat color genes were in the targeting plasmid. In an example, the blastocysts were obtained from C57BL/6 mice, which are solid black in color. Because the ES cell line E14TG2a was isolated from strain 129/Ola embryos, it and all cell lines derived from it were expected to carry the coat color markers characteristic of this mouse strain. These include the dominant AW allele at the agouti locus, the recessive chinchilla allele at the c-locus, and the recessive p-allele (pink-eyed dilution) at the p-locus. Contribution of ES cells to the mesoderm-derived portions of hair follicles results in an agouti coat. Hair follicles to which melanocytes of ES cell origin (and therefore carrying the P and c ch mutation) have migrated produce cream colored hairs. Both of these coat colors are easily distinguished from the solid black coat seen in pups derived from non-agouti C57BL/6 host blastocysts. The ES cell line D3 is derived from 129/SV and carries only the dominant Aw mutation. Chimerism is visualized by the presence of agouti coat color.

In an illustrative embodiment, three ES cell lines carrying an inactivated alpha gene were injected into C57BL/6 blastocysts. Chimeric animals as judged by coat color were obtained from all three lines. The following lines were injected: 66-154, 66-161, 66-168, 66-196, 67-

357. All cell lines produced chimeras when injected into mouse blastocysts, but germ line transmission of the ES cell genome was only achieved with cell lines 66-154, 66-168, and 66-196. Chimeras from line 66-161 were not test bred. Although cell line 67-357 produced numerous chimeras, the majority of highly chimeric males were sterile, and no transmission was obtained with this cell line.

Generation of Animals Heterozygous for the Mutated FcεRIα Locus

Chimeric animals are generated as described and bred to suitable mouse lines such as B6D2 or C57BL/6 mice(Koller and Smithies, 1989). Offspring carrying the targeted gene are identified by Southern analysis of tail DNA prepared as described (Miller et al., 1988). All the manipulations are performed on 4 to 8 week old mice that are FcεRI (+/+) and (-/-) littermates.

If the cells from any of the targeted cell lines contributed to the gonads of developing embryos, the resulting animals produce sperm that contain the ES cell genome, and thus are capable of passing the targeted mutation on to their offspring.

Offspring arising from eggs fertilized by sperm carrying the ES cell genome can be identified by markers such as coat color. In an illustrative embodiment, the ES cell genomes of all of the targeted lines are homozygous for the dominant color coat marker Aw. If the chimera is mated with an animal that is non-agouti, such as C57BL/6 or B6/D2, offspring that arise from sperm of ES cell origin will have agouti coats, while those derived from sperm of blastocyst origin will not. Using this criterion, it was determined that chimeras generated from two of the three targeted ES cell lines were able to transmit the ES cell genome to their offspring. By random chance, 50% of the resulting agouti offspring would be expected to inherit the mutated alpha gene. Animals carrying the mutant locus were identified by the analysis of DNA isolated from tail biopsies taken from

these animals.

Generation of Animals Homozygous for the Mutated FcεRIα Locus

Male and female animals whose DNA indicated that they were carrying one copy of the mutated FcεRI locus are mated. Tail biopsies are taken from offspring. DNA is prepared and analyzed by Southern blot for the presence of the mutated and wild type FcεRIα locus. One quarter of the animals analyzed are found to be homozygous for the mutation. Homozygotes then are analyzed for the affects of the mutation.

Passive Cutaneous Anaphylaxis is Prevented in FcεRI (-/-) Mice

To assess the contribution of FcεRI in allergic reactions, the *in vivo* model of passive cutaneous anaphylaxis (PCA) is suitable, pursuant to Wershil *et al.* (1987), in which local extravasation, fibrin deposition and tissue swelling is induced by local injection of IgE followed by intravenous antigenic challenge. Anti-DNP IgE (0.1 mg) or the same amount of anti-DNP IgG is injected in the right ear of both +/+ and -/- mice. As a control, the left ear of these mice is injected with the vehicle alone. After 20 hours, all animals are injected intravenously with [^{125}I]-fibrinogen, before being challenged with DNP_{30-40}-HSA injected with Evans Blue dye. The total fibrin deposit and the urea insoluble fraction corresponding to the cross-linked fibrin is quantitated in both ears of each experimental mouse.

In an illustrative embodiment, in +/+ animals, injection of IgE and antigen led to a fibrinogen/fibrin deposit in the right ear twice as large as the deposit in the left ear. Furthermore, the amount of cross-linked fibrin in the right ear was 4-5 fold the amount recovered from the control ear. In contrast, no differences between the right and left ears were detected in -/- mice injected with IgE. No effect was seen in either +/+ or -/- mice injected with IgG. Dye extravasation and swelling (13% weight increase) were detected only in the

right ears of +/+ mice injected with IgE and antigen. At the dose of 0.1 μg, therefore, IgE but not IgG is capable of inducing passive cutaneous anaphylaxis, and then only when FcεRI is present.

In the mice, having the FcεRI -/- phenotype appears not to engender any overtly undesirable features. The mice have no obvious propensity to autoimmune diseases, or susceptibility to infection, as would perhaps manifest if their immune systems were overtly altered. There is no apparent effect of the absence of FcεRI on B and T cell numbers or population subsets. The mast cells of these mice express reproducibly higher levels of FcγRII/III, but this does not seem to translate into an altered biological response. A possible explanation for this phenomenon is that the absence of competition by the FcεRIα chain is translated into an exclusive availability of γ chains for assembly with FcγRIII.

FcεRI (-/-) Mice are Resistant to Systemic Anaphylaxis

Another test for an allergic reaction involves inducing systemic anaphylaxis. For this test, IgE is injected intravenously 24h before intravenous injection of antigen. The antigenic challenge is immediately followed by a dramatic increase in vascular permeability, generalized fluid extravasation, and profound shock exhibited by a drop in blood pressure and body temperature.

To induce systemic anaphylaxis in an illustrative embodiment, both +/+ and -/- mice were injected intravenously with 20 mg of anti-DNP IgE or vehicle. Another set of mice was injected with 20 mg of mouse anti-DNP IgG or 400 mg of rabbit anti-TNP IgG. All mice were challenged 24h later with 1 mg of DNP_{30-40}-HSA. Evans Blue dye was concomitantly injected with antigen to visualize the fluid extravasation. A significant drop in body temperature occurred in the IgE but not the IgG injected FcεRI +/+ mice. This drop reached a maximum after about 25 min and was accompanied by obvious tachycardia, piloerection and prostration.

Rectal temperatures of FcεRI (+/+) and FcεRI (-/-) mice were taken during IgE induced systemic anaphylaxis. Six FcεRI (+/+) and 4 FcεRI (-/-) animals received 20 μg of anti-DNP IgE intravenously. One FcεRI (+/+) and 2 FcεRI (-/-) animals received 20 μg of murine anti-DNP IgG. Three FcεRI (+/+) and 3 FcεRI (-/-)animals received 400 μg of rabbit anti-TNP IgG. All the animals received 1 mg of DNP_{30-40}-HSA in 0.5 % Evans Blue i.v. 24h later. Four additional FcεRI(+/+) mice received antigen only. The monitoring of the rectal temperature was started at the time of antigen injection.

Dye extravasation in FcεRI (+/+) and FcεRI (-/-) mice was examined during IgE induced systemic anaphylaxis. Mice received 20 μg of anti-DNP IgE intravenous and 24 hours later, 1 mg of DNP_{30-40}-HSA in 0.5% Evans Blue intravenous. Photographs of the mice were taken 60 minutes after antigen challenge.

The drop in temperature and other symptoms were completely absent in -/- mice. The anaphylactic shock was best visualized by the extravasation of the dye, which was marked in the ears and feet of the FcεRI +/+ mice and absent in the FcεRI-deficient mice.

Serum Clearance of IgE

IgE is iodinated using chloramine T to a specific activity of 4X105 cpm/mg. Twenty or 1 mg of [125I]-IgE in 200 ml PBS with 0.1 % BSA are injected intravenously into +/+ and -/-mice. Blood is collected in heparinized tubes at various intervals of time. To separate the[125I]-IgE from free [125I]-iodine or degradated products, [125I]-IgE in blood samples (50 ml) is immuno precipitated with rabbit anti-murine IgE antibodies bound to protein A-sepharose resin (Pharmacia). After centrifugation for 5 minutes at 1500 g, radioactivity of the pellet is counted. Animals are sacrificed after 24 hours. Organs (ear, skin, tongue, liver, spleen, stomach, kidney and trachea with thyroid) are taken and weighed, and radioactivity is counted.

Whether the absence of FcεRI has an influence on the

clearance of IgE from the serum, following an intravenous injection of IgE under conditions similar to those used in the systemic anaphylaxis protocol, is determined as follows. With an intravenous injection of 20 mg of [125I]-IgE, the serum clearance over 24h appeared similar in both +/+ and -/-mice. The apparent half-life was about 2 hours during the 3 hours following injection and increased to around 8 hours. The data obtained from normal mice were consistent with those from Haba *et al.* (1985). After 24 hours, there was still a substantial amount (0.7 mg/ml) of circulating IgE in the serum. Furthermore, comparable levels of radioactivity/mg of tissue were found in various organs from +/+ and -/- mice. Similar results were obtained when only 1 mg of [125I]-IgE was injected. Therefore, at the time of antigenic challenge, the serum level of IgE was similar in both -/- and +/+ mice. This implied that the capacity to form IgE-IC, which could bind to low affinity IgE receptors such as FcγRII/III, was equivalent in both wild type and FcεRI-deficient mice.

Serum clearance of IgE following intravenous injection was unexpected. As the only high-affinity IgE receptor, FcεRI apparently does not act as the principal reservoir for IgE. The clearance of IgE in FcεRI-deficient mice parallels perfectly the pattern observed in normal mice, whether 20 μg or 1 μg of IgE are injected. FcεRI therefore seems to have little or no role in the sequestration of IgE. This observation is consistent with the suggestion that CD23/FcεRII is the principal binding structure for IgE and affects IgE homeostasis (Chen, 1991).

Production of a Transgenic Mouse With a Human Gene Encoding a Chain of FcεRI Replacing its Murine Homologue

As aspect of the present invention is a mouse that is useful in the analysis of potential inhibitors of allergic responses in humans. To this end a mouse line is created, according to the present invention, in which the crucial structural proteins involved in allergic

responses are replaced by their human counterparts. Creation of such a mouse line requires three steps. The first of these is the identification and characterization of proteins that play an important role in allergic responses. Such proteins represent reasonable targets for potential inhibitors of these responses. The second step is the inactivation of genes in the mouse genome that encode the proteins identified in step one. And the final step is the replacement of the genes inactivated in step two with their human homologous counterparts.

Previous research indicates that the FcεRI receptor is one of the proteins that plays an important role in allergic responses. To initiate creation of the mouse line described above, the murine gene encoding this receptor is replaced with the equivalent human gene. The first step in this process is to inactivate the FcεRI receptor in the mouse genome via homologous recombination in mouse embryonic stem (ES) cells. Other inactivation methods are available, such as site-directed mutagenesis. Analysis of animals that are homozygous for the inactivated FcεRI gene, and therefore totally unable to produce the FcεRI receptor, as related herein has confirmed the importance of this receptor in both passive and active anaphylaxis in mice.

The final step in production of mice expressing only human FcεRI receptors is the substitution of a human gene for its murine homologue in the genome of FcεRI deficient mice. To accomplish this step, the human FcεRI gene is introduced into the mouse genome using one of four different approaches: (1) random integration using conventional transgenic techniques, (2) random integration via ES cells, (3) targeted integration into the hprt locus via ES cells, and (4) targeted replacement of the endogenous FcεRI gene via ES cells. These various alternatives, are suitable to develop a mouse strain in which both the level and pattern of expression of the FcεRI gene mimic those seen in humans. This animal is useful for assessing the efficiency of various

inhibitors.

1. Random Integration of the FcεRI Gene Into the Mouse Genome Using Conventional Transgenic Techniques: Methods of producing mice with transgenes added to the genome are described in U.S. Pat. Nos. 4,736,866, 5,175,383 and 5,175,385. A standard technique for the addition of genes from the human genome to mice is injection of 1 day embryos with a fragment of DNA containing the gene and the necessary regulatory sequences. Using this technique, mice are obtained in which the FcεRI gene plus appropriate regulatory sequences have integrated at random sites in the genome. Crossing of these animals with mice homozygous for the disrupted FcεRI gene results in animals carrying the transgene and heterozygous for the targeted gene. A subsequent round of breeding is necessary to yield animals homozygous for the targeted mutation and therefore defective in the production of mouse FcεRI. Because of the presence of the transgene, however, these mice express a humanized IgE receptor. To shorten the time of these experiments, 1-day embryos are obtained that are from FcεRI deficient animals, and test breeding is carried out by mating founders to FcεRI deficient animals.

The fragment introduced into the 1-day embryos is of two types. The first contains a large segment of upstream DNA 5' to the human alpha gene. This fragment is sufficient to direct the cell type specific synthesis of this gene. In addition a construct in which the human alpha gene is driven by a suitable promotor, either the murine promoter for the alpha chain gene, CMV, HTVL1, or the like, is introduced into 1-day embryos. While expression in a large number of cell types is expected, this should include those in which binding of IgE triggers anaphylaxis.

The limitation of this method is that integration occurs at random locations in the genome and is usually preceded or concomitant with numerous duplication events.

The unpredictability of the site of integration and number of copies of the transgene, combined with the fact that elements very distant to the gene may influence its expression, has made it difficult to mimic the expression of native genes using this method. Some examples of success are available in the art, however, although these usually entail extensive work to identify such upstream elements or the use of large fragments of DNA such as YACs (yeast accessary chromosome). In the case of the latter, introduction into 1-day old embryos becomes more difficult. YACS may be introduced into mouse lines via ES cells. The use of ES cells to obtain random integration of foreign DNA into the mouse genome is described in more detail in the following section.

2. Introduction of the Human FcεRI Gene Into the Mouse Genome Via Random Integration in ES Cells: By this method, the human FcεRI gene is introduced into ES cells via electroporation or calcium phosphate precipitation, either in the presence of DNA carrying a selectable marker gene or as part of a construct that contains the marker gene. ES cells that have incorporated DNA as demonstrated by their growth in selectable media are then screened for the presence of the human FcεRI gene. These ES cells are then introduced into mouse blastocysts. Resulting female chimeras are sacrificed and analyzed for the expression of the human FcεRI, taking into account the relative contribution of ES cells to various tissues. Males from those lines which display appropriate levels and patterns of expression levels are then tested for germ line transmission.

Two different marker genes, the neomycin resistance gene and the hprt gene, are suitable for initial selection of the ES cells carrying the transgene in this scheme. The neomycin gene is small and can easily be added to a construct carrying the human FcεRI gene. This ensures that virtually all of the ES cell colonies that are resistant to neomycin carry the alpha gene. On the other hand, the hprt gene has the advantage of allowing

use as a starting population of the ES cell line carrying one disrupted mouse FcεRI locus. While this line has become resistant to neomycin as a result of manipulation to target this locus, it originates from an ES cell line that carries a spontaneous mutation in the endogenous hprt locus, thus allowing the hprt gene to be used to identify cells that have integrated DNA into their genome. This means one generation of breeding time can be eliminated in producing animals that express only the human FcεRI gene.

Introduction of the human FcεRI gene into the mouse genome via ES cells overcomes some of the disadvantages of random integration by allowing analysis of expression, first at the level of the ES cells and second at the level of the chimera, before commitment to breeding a given line. However, it may be necessary to use a method which allows more control over the expression of the human FcεRI gene. Because deviation of the expression of the transgene from that of its endogenous counterpart is usually attributed to variations in copy number and the random nature with which the transgene integrates into the endogenous genome, another suitable system is described in more detail in the next section, which is designed to limit these variables.

3. Introduction of a Single Human FcεRI Gene Into a Predicted Location in the Mouse Genome Via ES Cells: In this system the human alpha gene is inserted into a construct designed to correct the hprt mutation in the ES cell line E14tg2a. The outcome of this correction event is that a single copy of the transgene is located on the X chromosome. It is likely that a single copy of the gene in one location results in expression that more closely mimics the native state. This situation also makes it easier to predict and compare the outcome of experiments designed to fine tune levels of expression.

4. Replacement of the Mouse FcεRI Gene with the Human Equivalent by Targeted Integration in ES Cells: Another suitable strategy is to use homologous recombination to replace the coding sequences of the mouse FcεRI gene, with the coding sequences from the corresponding human gene. This places the human gene under the same regulatory constraints as the endogenous gene. The high targeting frequency that has been demonstrated for this locus in the present invention makes such an approach desirable.

Candidate Inhibitor

In still further embodiments, the present invention concerns a method for identifying an inhibitory compound which interferes with an IgE-mediated allergic reaction. These "candidate substances" are first screened *in vitro*. It is contemplated that inhibitors will be small, charged molecules, although the invention is not limited to detecting these compounds.

After determining a candidate substance by *in vitro* screening, the substance is introduced into the system of a transgenic mouse in which a human gene encoding at least one chain of the FcεRI replaces its murine homologue. The substance is generally introduced by intravenous injection of the substance in a suitable carrier or diluent.

Suitable control mice are preferred, including a transgenic mouse of the present invention injected with a control substance, for example, only the carrier or diluent. Other controls include mice transgenic for other human genes.

An identical environment is provided to control and treated mice to induce an allergic reaction. Suitable methods are required to determine whether an allergic reaction occurred. Absence of an allergic reaction in the treated, but not the control mice, provides evidence that the candidate substance is inhibitory.

The substance to be tested should be in a relatively purified form, to assay for the effect of the substance

to be tested not effects caused by impurities.

A range of concentrations of the candidate substance is tested to avoid a false negative result arising from concentrations that are too low, and to estimate effective concentrations to test in clinical trials of candidate substances that are inhibitory in the transgenic mice tests.

EXAMPLES

The following are examples characterizing the effects of disrupting the mouse gene encoding the α chain of the FcεRI.

Example No. 1:

Mast Cell Development in FcεRIα (-/-) Mice

It has been speculated that FcεRI plays a role in mast cell development. This speculation was based on the early appearance of FcεRI on precursors of mast cells (Thompson *et al.*, 1990; Ashman *et al.*, 1991) and on the capacity of FcεRI to mediate synthesis and release of mast cell growth factors such as interleukin-3. Investigations using FcεRI deficient mice shows that FcεRI does not play an important role in mast cell development. The absence of FcεRI did not affect the number or appearance of mast cells in any of the tissues and organs examined. In addition, mast cells appeared to function normally when stimulated by non-specific mast cell activators.

Mast cell development appears normal in FcεRIα (-/-) mice. Mast cells from 4 and 8 week old mice were examined in histological sections of different tissues (ear skin, dermis of back skin, tongue, tracheal lamina propria, submucosa of glandular stomach, and intestinal submucosa). No morphological differences between the mast cells of FcεRIα -/- and +/+ animals of the same litter could be detected. Furthermore, the number of mast cells in six different organs known to be rich in mast cells was equivalent in -/- and +/+ animals (Table 1). Thus, the development and differentiation of mast cells does not seem to be affected by the absence of a

functional FcεRIα gene.

Table 1: Number of Mast Cells per square mm

Organ	FcεRI (+/+)	FcεRI (-/-)
Ear	156.27 ± 23.57	151.08 ± 47.78
Skin	50.51 ± 12.35	42.14 ± 3.00
Tongue	34.28 ± 6.20	31.31 ± 7.99
Trachea	15.54 ± 6.20	18.19 ± 4.80
Stomach	16.00 ± 6.70	18.46 ± 9.35
Intestine	1.89 ± 0.58	1.87 ± 0.45

Mast cells per square millimeter in ear skin, dermis of back skin, tongue, tracheal lamina propria, submucosa of glandular stomach and intestinal submucosa. Values are means +/- S.E.M. FcεRI(+/+) (n=5), FcεRI(-/-) (N=4).

Example No. 2:

Expression of FcεRI and FcεRII/CD23 on Mast Cells and B Cells

To insure that disruption of the FcεRIα gene abolished FcεRI expression, FcεRI expression on bone marrow derived mast cells (BMMC) was analyzed. The total number of mast cells from FcεRI -/- and +/+ animals after 3 weeks in culture in the presence of IL-3 was comparable. Those cells were analyzed by FACS after double staining with anti c-kit receptor antibody and monomeric IgE.

Bone marrow derived mast cells (BMMC) were stained with biotinylated monomeric IgE and streptavidin-phycoerythrin and with anti c-kit receptor antibody and goat $F(ab')_2$ anti-rabbit IgG-FITC. Splenocytes were stained with biotinylated IgE and streptavidin-phycoerythrin and FITC-labeled goat anti-mouse immunoglobulins. In both FcεRI -/- and +/+ mice, most of the cells expressed the c-kit receptor, as expected for mast cells. Binding of monomeric IgE was

present in FcεRI +/+ and absent in -/- animals. Therefore disruption of the FcεRIα gene suppressed FcεRI surface expression and the binding of monomeric IgE.

Spleen B cells constitutively expressing FcεRII/CD23 (Conrad, 1990) were also analyzed by double staining with monomeric IgE and an antibody against surface immunoglobulins. In both +/+ and -/- mice, the population of B cells appeared normal and virtually all B cells showed IgE binding. This binding was due to CD23 because, unlike the binding of monomeric IgE to BMMC, it was completely inhibited by anti-CD23. Thymocytes were also analyzed for various markers, including TCRαß, TCRγδ, FcγRII/III, CD5, CD3, CD4 and CD8 and for their capacity to bind monomeric IgE. The thymocytes from both +/+ and -/- mice did not bind IgE and their various markers of differentiation were qualitatively and quantitatively normal.

Example No. 3:

Increased Expression of FcγRII/III in FcεRI (-/-) Mast Cells

The homologous extracellular domains of FcγRII and FcγRIII react equivalently with the monoclonal antibody 2.4G2 (Ravetch and Kinet, 1991). Therefore, BMMC were stained with 2.4G2 to detect expression of FcγRII/III. Binding of IgE-immune complexes and oligomeric IgG to FcγRII and FcγRIII on BMMC of FcεRI (+/+) and FcεRI (-/-) animals. Expression of FcγRII/III was exhibited using 2.4G2-FITC. The binding of oligomeric IgG was revealed by using goat $F(ab')_2$ anti-rabbit IgG-FITC in the absence or presence of 2.4G2. Binding of IgE-immune complexes was displayed in the absence or presence of monomeric IgE or of both IgE and 2.4G2. The cells analyzed were from a pool of cultured bone marrow mast cells obtained from 2 mice.

Unexpectedly, this expression was reproducibly higher in -/- mice, when compared with the +/+ littermates. In one representative experiment, the mean and median fluorescence intensity were respectively 509 and 469 in

+/+ mice and 561 and 554 in -/- mice. This was confirmed by direct binding of oligomeric IgG which was completely inhibited with 2.4G2.

Example No. 4:

FcγRII/III on Mast Cells is an IgE Receptor

The capacity of FcγRII/III to bind IgE-immune complexes (IgE-IC) was analyzed. Binding of IgE-immune complexes and oligomeric IgG to FcγRII and FcγRIII was detected on BMMC of FcεRI (+/+) and FcεRI (-/-) animals. Expression of FcγRII/III was exhibited using 2.4G2-FITC. The binding of oligomeric IgG was revealed by using goat $F(ab')_2$ anti-rabbit IgG-FITC in the absence or presence of 2.4G2. Binding of IgE-immune complexes was displayed in the absence or presence of monomeric IgE or of both IgE and 2.4G2. The cells analyzed were from a pool of cultured bone marrow mast cells obtained from two mice. In +/+ mice, IgE-IC bound principally to FcεRI as shown by inhibition with unlabelled monomeric IgE. However, the addition of 2.4G2 further inhibited the binding of IgE-IC. This confirms a previous observation that IgE-IC binds to mouse FcγRII/III (Takizawa *et al.*,1992). Evidence for this was more striking in -/- mice. Monomeric IgE competed poorly with the binding of IgE-IC. Only 2.4G2 was able to compete efficiently with this binding. Thus, BMMC of the -/- mice bind IgE-IC via FcγRII/III.

Example No. 5:

Serotonin Release by Activated Mast Cells (BMMC)

BMMC were loaded with [^{3}H]serotonin, incubated with monomeric anti-DNP IgE, and then challenged with various amounts of the multivalent antigen dinitrophenol [30-40]-human serum albumin (DNP_{30-40}-HSA). A dose-dependent release of up to 40% of the total cellular serotonin was observed with the BMMC from +/+ but not -/- mice. However, -/- BMMC were capable of releasing serotonin after stimulation with ionomycin and therefore were functionally intact. Engagement of FcγRII/III by cross-linking with 2.4G2 on +/+ or -/-BMMC did not lead

to detectable serotonin release. This result was consistent with previous reports that BMMC cultured in presence of IL-3 cannot be triggered through FcγRII/III (Katz *et al.*, 1992). However, peritoneal mast cells from both +/+ and -/- mice could release endogenous histamine after cross-linking of FcγRII/III with 2.4G2.

MATERIALS AND METHODS

Histological Analysis

Mast cells were stained with acidic toluidine blue (pH 1.5), Giemsa or alcian blue-safranin. The number of mast cell per square millimeter was determined. For each tissue, twenty-five microscopic fields were counted on five non-serial sections.

Flow Cytometry

Bone marrow derived mast cells were isolated from 4 to 8 week old mice according to Rottem et al. (1992). Samples (2 to 5X105 cells) of bone marrow derived mast cells,thymocytes or splenocytes were analyzed by flow cytometry. FcεRI was detected using biotinylated mouse monoclonal anti-DNP IgE (20 mg/mL) and streptavidin-phycoerythrin(10 mg/mL) (Molecular Probes Inc.) after preincubation with a rat anti-murine FcγRII/III(2.4G2) monoclonal antibody (10 mg/mL). Unstained cells, cells stained with streptavidin-phycoerythrin alone, or cells preincubated with a 10-fold excess of unlabeled IgE were used as controls. Interaction of IgE with FcεRII (CD23) was assessed by inhibition of binding of biotinylated IgE with a rat anti-murine CD23 monoclonal antibody (B3B4) (10mg/mL) (Pharmingen) after preincubation with 2.4G2 (10 mg/mL). Presence of FcγRII and FcγRIII was detected using 2.4G2-FITC (10 mg/mL). Binding of IgE-antigen immune complexes and of oligomeric IgG to FcγRII/III were performed as described before (Takizawa et al., 1992). Mast cells were identified using rabbit polyclonal anti-murine c-kit receptor antibody (gift from Dr. Peter Bessmer, Memorial Sloan-Kettering Cancer Center, New York) and goat F(ab')2 anti-rabbit IgG-FITC (10 mg/mL)

(Jackson Immuno Research). B cells were identified by detection of surface immunoglobulins using goat anti-mouse Ig-FITC (10 mg/mL) (Pharmingen).

Serotonin and Histamine Release Assay

Bone marrow derived mast cells were incubated overnight in the presence of 1 mCi/mL of 5-[1,2-3H(N)]-hydroxytryptamine binoxalate ([3H]-serotonin) (DuPont), then washed twice and resuspended for triggering at a density of 106 cells/mL as described before (Takizawa *et al.*, 1992). Radioactivity was determined separately in the pellet and in the supernatant. Percent release was calculated by dividing cpm in the supernatant by total cpm. Histamine assay was performed according to Rottem et al. (1992).

CITED DOCUMENTS

The documents listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques or compositions employed according to the present description.

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WHAT IS CLAIMED IS:

1. An *in vivo* assay for an allergic response, comprising:

(a) providing a transgenic mouse that expresses at least one human gene encoding a chain of the FcεRI receptor and wherein the homologous mouse gene is inactive, and

(b) subjecting said transgenic mouse to conditions conducive to an allergic reaction, and then

(c) detecting whether said allergic reaction occurred.

2. The assay of claim 1, wherein said human gene encodes the alpha chain of the FcεRI receptor.

3. The assay of claim 1, wherein the allergic response is systemic anaphylaxis.

4. A mouse having a genome in which (i) at least one endogenous gene encoding a chain of the murine FcεRI is inactivated and (ii) a human gene encoding a chain of the human FcεRI is integrated and expressed.

5. The mouse of claim 4, wherein said human gene encodes the alpha chain.

6. A mouse wherein at least one endogenous gene encoding a chain of the FcεRI is inactivated.

7. An *in vivo* method to screen for an agent that inhibits an allergic response in a human, comprising:

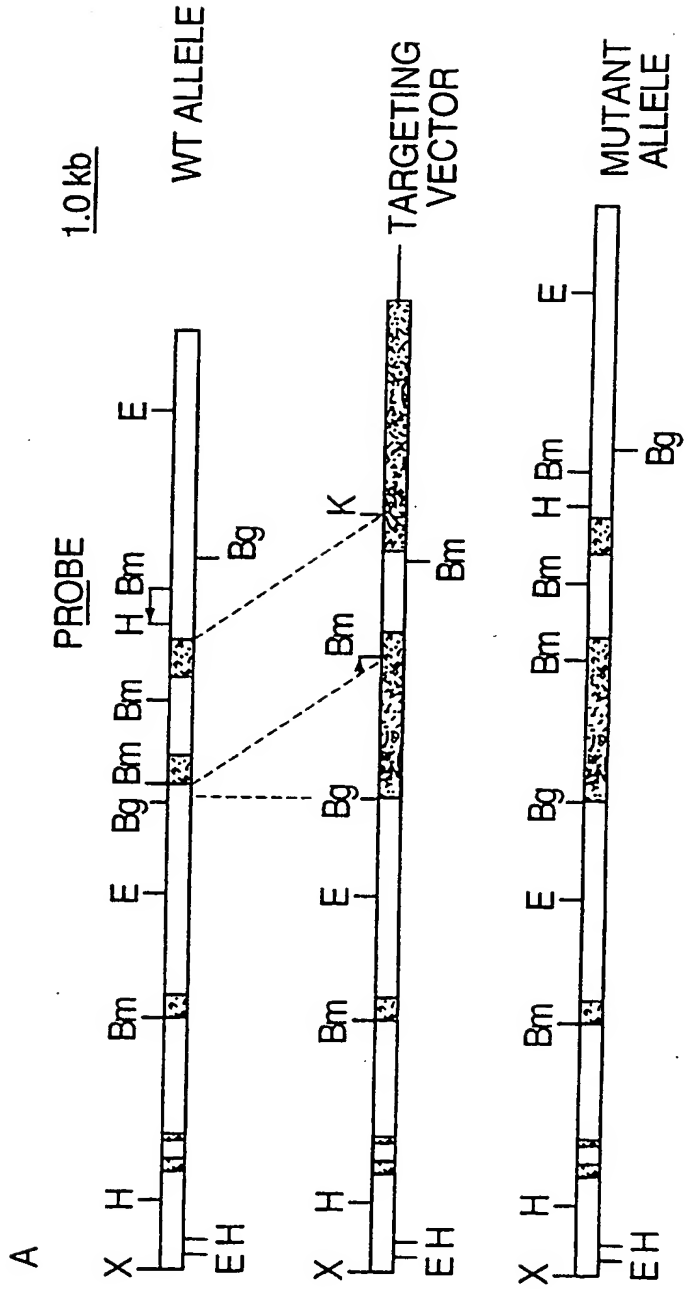
(a) exposing a humanized transgenic mouse to said agent, wherein said transgenic mouse expresses at least one human gene encoding for a chain of the FcεRI,

(b) subjecting said transgenic mouse to conditions conducive to an allergic reaction and then

(c) determining whether an allergic reaction occurs, whereby any inhibition of said allergic reaction by said agent is gauged.

8. A method of affecting an allergic response, comprising treating a patient with an amount of an inhibitor according to claim 7 that is sufficient to inhibit said response.

FIG. 1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